GGA proteins regulate retrograde transport of BACE1 from endosomes to the trans-Golgi network

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Golgi-localized, γ ear-containing, ADP ribosylation factor-binding (GGA) proteins have been shown to be implicated in the sorting of cargo proteins from the trans-Golgi network (TGN) to endosomal compartments. GGAs directly bind to DXXLL motifs in the cytoplasmic domains of cargo proteins. The Alzheimer-associated β-secretase BACE1 also interacts with GGA proteins, but the functional relevance of this interaction was unknown. Here, we show that GGA1 regulates the retrograde transport of internalized BACE1 from endosomal compartments to the TGN by direct interaction in a phosphorylation-dependent manner. While phosphorylated BACE1 is efficiently transported from endosomes to the TGN, non-phosphorylated BACE1 enters a direct recycling route to the cell surface. Our data indicate that GGA proteins are not only involved in the sorting at the TGN but also mediate the retrograde transport of cargo proteins from endosomes to the TGN.

Introduction

The generation of the Alzheimer’s disease-associated amyloid β-peptide (Aβ) involves sequential cleavages of the β-amyloid precursor protein (βAPP) by proteases called β- and γ-secretase (Annaert and de Strooper, 2002; Selkoe, 2001; Walter et al., 2001a). β-Secretase cleaves βAPP at the N-terminus of the Aβ domain resulting in the secretion of the βAPP ectodomain and the generation of a membrane bound C-terminal fragment (CTF). The subsequent cut of the CTF by γ-secretase finally liberates Aβ. β-Secretase was identified as the aspartyl protease BACE1, a type I membrane protein with the catalytic domain directed to luminal compartments, a single transmembrane domain and a small cytoplasmic domain (Haniu et al., 2000; Hussain et al., 1999; Lin et al., 2000; Sinha et al., 1999; Yan et al., 1999). In addition to βAPP, BACE1 also cleaves the βAPP-like proteins (APLP)-1 and APLP-2 (Li and Sudhof, 2004) and a Golgi-resident sialyltransferase (Kitazume et al., 2001).

BACE1 is transported from the endoplasmic reticulum (ER) to the Golgi where it matures by complex N-glycosylation and proteolytic removal of the pro-peptide (Capell et al., 2002; Creemers et al., 2000). After maturation, BACE1 is further transported to the cell surface from where it can be reinternalized into endosomal compartments (Huse et al., 2000; Walter et al., 2001b). Consistent with these findings, β-secretase activity has been localized to the TGN and endosomal compartments as well as to the plasma membrane (Vassar and Citron, 2000; Walter et al., 2001a). We and others found that BACE1 can be phosphorylated within its cytoplasmic domain by casein kinase-1 (Pastorino et al., 2002; Walter et al., 2001b). The expression of phosphorylation site mutants indicated that phosphorylation affects the subcellular localization of BACE1 (Walter et al., 2001b). However, the molecular mechanisms underlying this effect have not been identified.

BACE1 contains a characteristic binding motif within its cytoplasmic domain for GGA proteins (Fig. 1A), and direct binding of BACE1 and GGA proteins has been demonstrated recently (He et al., 2002, 2003; Shiba et al., 2004; von Armim et al., 2004). However, a functional implication of this interaction has not been demonstrated. GGA proteins are monomeric adaptor proteins that are known to mediate transport of cargo proteins from the TGN to endosomes (Dell’Angelica et al., 2000; Doray et al., 2002a; Hirst et al., 2000) and consist of a N-terminal Vps, Hrs and STAM (VHS) domain, a GGA and Tom (GAT) domain, a variable hinge region and a γ-adaptin ear (GAE) domain. The VHS domain binds to DXXLL motifs in cytoplasmic domains of cargo proteins (Fig. 1A). The GAT domain and the hinge region bind to ADP-ribosylation factors (ARFs) and clathrin, respectively, while the GAE domain interacts with additional proteins (Bonifacino, 2004). Cargo proteins that are recognized by VHS domains include the cation-dependent and -independent mannose 6-phosphate receptors (MPRs), sortilin and LRP3 (Bonifacino, 2004; Ghosh et al., 2003a). So far, three distinct GGA proteins (GGAl, GGA2, GGA3) have been identified that...
might exert distinct functions in protein sorting, although overlapping subcellular localization and functional redundancy has been demonstrated (Bonifacino, 2004; Robinson, 2004).

In this study, we sought to determine the functional role of GGA1 in the regulation of the phosphorylation-dependent transport of BACE1. We found that both proteins colocalize in the TGN and endosomal compartments and directly interact in a phosphorylation-dependent manner. Our data also demonstrate that, in addition to their function in forward transport of cargo proteins from the TGN to endosomes, GGA proteins are also implicated in the retrograde trafficking of BACE1 from endosomes to the TGN.

**Results**

The retrograde transport of BACE1 from endosomes to the TGN is dependent on the phosphorylation state of Ser498 in its cytoplasmic domain (Walter et al., 2001b). While phosphorylated BACE1 is efficiently transported from the cell surface to the TGN, non-phosphorylated BACE1 accumulates in endosomal compartments. We used HEK 293 cells stably overexpressing BACE1 wt or phosphorylation site mutants S498A or S498D (Fig. 1A) to investigate whether phosphorylation also affects the internalization of BACE1 from or recycling to the cell surface by biontinylation assays. The internalization of BACE1 from the cell surface was found to be independent of the phosphorylation state, as indicated by similar rates of endocytosis of BACE1 wt and the two phosphorylation site mutants (Figs. 1B–D).

The amount of biontin-labeled BACE1 internalized after 15 min was set as 100%. Values represent means of three independent experiments ± SD. The rates of recycling differ significantly between the different BACE1 variants (*P < 0.05; **P < 0.01; ***P < 0.005 (ANOVA test)).
It has been shown that the cytoplasmic domain of BACE1 can bind to proteins of the GGA family in vitro and in cultured cells (He et al., 2002, 2003; Shiba et al., 2004; von Arnim et al., 2004). To assess a potential functional role of GGA1 in the subcellular trafficking of BACE1, we generated HEK 293 cells that stably overexpress both proteins and first analyzed the subcellular localization of BACE1 and GGA1 by confocal microscopy. In addition to BACE1 wt, we also expressed the phosphorylation site mutants S498A and S498D (Walter et al., 2001b). All variants of BACE1 were localized in juxtanuclear structures, cytoplasmic vesicles and at the plasma membrane (Fig. 2A). As compared to BACE1 wt and the S498D mutant, the S498A mutant which lacks the phosphorylation site revealed pronounced localization within cytoplasmic vesicles (Fig. 2A). Consistent with previous studies (Dell’Angelica et al., 2000; Hirst et al., 2000; Shiba et al., 2004), GGA1 was also detected in juxtanuclear structures and in cytoplasmic vesicles, where it showed significant colocalization with BACE1 wt and the phosphorylation site mutants (Fig. 2A). To characterize the juxtanuclear structures that contain BACE1 in more detail, cells were costained with antibodies against BACE1 and TGN46, a protein residing in the TGN. BACE1 and TGN46 showed significant colocalization in the juxtanuclear structures, indicating a localization of BACE1 in the TGN (Fig. 2B). Taken together, these data indicate an association of BACE1 and GGA1 in the TGN and in cytoplasmic vesicles.

We next characterized the subcellular compartments involved in the retrograde transport of BACE1 by double staining with markers for early endosomes (EEA1) and the TGN (TGN46), respectively. Cell surface-located BACE1 was labeled with antibody 7523 and allowed to internalize for 10 and 30 min respectively. After internalization from the cell surface for 10 min, BACE1 was detected in peripheral cytoplasmic vesicles that were partly positive for EEA1, indicating localization of BACE1 in early endosomal compartments (Fig. 3A). Notably, internalized BACE1 also colocalizes with GGA1 in these compartments (Fig. 3B). After longer incubation times (30 min), BACE1 was predominantly found in juxtanuclear structures that were positive for TGN46 (Fig. 3C). Together, these data indicate that BACE1 associates with GGA1 in endosomal compartments during retrograde transport from the cell surface to the TGN.

To demonstrate that GGA1 directly interacts with BACE1, we performed pull-down assays with a fusion protein of GST...
and the GGA1 VHS domain (GST-GGA1 VHS). GST-GGA1 VHS efficiently precipitated all three BACE1 variants from lysates of stably expressing cells (Fig. 4A). Likewise, a fusion protein carrying the C-terminus of BACE1 (GST-BACE1-CT) bound to a myc-tagged GGA1 variant that was stably expressed in HEK 293 cells (Fig. 4B). In contrast, a similar fusion protein carrying the cytoplasmic domain of BACE2 did not pull-down GGA1 (data not shown), consistent with the lack of a DXXLL motif in BACE2 (Fig. 1A). Since the fusion protein MBP-GGA1 VHS also interacts with GST-BACE1-CT in vitro (Fig. 4C), no additional cellular factors are necessary for the interaction of both proteins. These data confirm and extend recent studies (He et al., 2002, 2003; Shiba et al., 2004; von Arnim et al., 2004) and indicate that GGA1 specifically binds to the cytoplasmic domain of BACE1. As determined by surface plasmon resonance (SPR) analysis, we found that phosphorylation of BACE1 at Ser498 significantly increases this interaction more than 25-fold (Fig. 5). While the non-phosphorylated cytoplasmic domain of BACE1 binds to GST-GGA1 VHS with a dissociation constant \(K_D\) of 24 \(\mu\)M, a \(K_D\) of 0.9 \(\mu\)M was determined for the complex of GST-GGA1 VHS and the phosphorylated cytoplasmic domain of BACE1.

These findings raise the question whether GGAs are functionally involved in the phosphorylation-dependent retrograde traffick-
ing of BACE1 from endosomes to the TGN. To address this, we compared the transport of BACE1 from endosomes to the TGN in cells expressing GGA1 wt or a truncated variant of GGA1 that contains only the VHS and GAT domains (GGA1 DN). Since this variant lacks the hinge region and the GAE domain, it does not bind to clathrin or other coat proteins on target vesicles and acts as a dominant-negative form of GGAs that interferes with the sorting of cargo proteins containing a DXXLL motif (Lefrancois et al., 2003; Puertollano et al., 2001). Consistent with previous reports (Doray et al., 2002b; Lefrancois et al., 2003), GGA1 DN was predominantly localized in juxtanuclear structures indicative for the Golgi and TGN (Fig. 6A). We also observed some GGA1 DN localization in peripheral vesicles and the cytoplasm, likely due to impaired association with membrane-bound clathrin (Fig. 6A). To investigate whether retrograde trafficking of BACE1 is affected by GGA1, we analyzed the subcellular localization of BACE1 30 min after uptake from the cell surface by immunocytochemistry. In cells expressing GGA1 wt, internalized BACE1 showed prominent juxtanuclear localization after 30 min (Fig. 6B, upper panel). In contrast, in GGA1 DN expressing cells, BACE1 revealed predominant localization in cytoplasmic vesicles (Fig. 6B, lower panel), indicating that GGA1 DN attenuated the retrograde transport of BACE1 from endosomal compartments to the TGN. Quantitative analysis of BACE1 localization proved that expression of GGA1 DN significantly affected the retrograde transport of BACE1 from endosomes to the TGN (Figs. 6C, D).

Discussion

The role of GGA proteins has been well characterized in the sorting of the MPRs from the TGN to endosomal compartments

Fig. 4. Interaction of BACE1 and GGA1. (A) Lysates of HEK 293 cells expressing BACE1 were incubated with GST-GGA1 VHS domain pre-bound to GSH-sepharose. GST was used as a negative control. Precipitated BACE1 was detected by Western immunoblotting with antibody 7520. (B) Lysates from HEK 293 cells overexpressing a myc-tagged GGA1 variant were incubated with the fusion protein GST-BACE1 CT or GST alone and precipitated with GSH-sepharose. Precipitated GGA1 was detected by Western immunoblotting with anti-c-myc antibody. (C) A fusion protein of MBP and GGA1-VHS domain (MBP-GGA1VHS) was incubated with GST-BACE1 CT wt and precipitated with GSH-sepharose. GST alone was used as negative control. Precipitates were analyzed by Western immunoblotting with an antibody against MBP.

Fig. 5. Phosphorylation-dependent binding of BACE1 and GGA1. (A–C) Surface plasmon resonance analysis of the interaction of BACE1 and GGA1. (A) Synthetic peptides representing the cytoplasmic tail of BACE1 were immobilized on a Biacore sensor chip. GST (0.5 mM) or GST-GGA1-VHS (0.5 mM) was injected (arrowheads indicate injection time points), and responses were recorded. (B, C) Sensograms of the interaction of GST-GGA1-VHS (concentrations ranging from 50 nM–5 μM) with non-phosphorylated (B) or phosphorylated (C) cytoplasmic domains of BACE1. The dissociation constants were calculated as 24 μM and 0.9 μM for the non-phosphorylated and phosphorylated peptides, respectively.
At the TGN, GGA1 binds preferentially to phosphorylated MPRs and targets these proteins to AP-1 positive vesicles destined for endosomes (Doray et al., 2002a; Puertollano et al., 2001). However, GGA proteins have also been detected in peripheral vesicular and endosomal compartments where they might exert additional functions. Recently, it was also demonstrated that GGA proteins recognize ubiquitinated CI-MPR and regulate endocytic trafficking and turnover of this protein in lysosomal compartments (Puertollano and Bonifacino, 2004; Scott et al., 2004).

Here, we demonstrate that GGA1 directly interacts with BACE1 and that both proteins colocalize in endosomal compartments and the TGN. Consistent with previous studies (He et al., 2002, 2003; Shiba et al., 2004; von Arnim et al., 2004), we also found that phosphorylation of the cytoplasmic domain of BACE1 strongly increases the binding to the VHS domain of GGA1. To characterize the functional role of BACE1 phosphorylation in endocytic trafficking, we used cells stably transfected with BACE1 wt or the phosphorylation site mutants S498A and S498D, respectively. This strategy has the major advantage that the functional role of phosphorylation can be investigated without pharmacological manipulation of kinase and/or phosphatase activities that might cause multiple side effects (Egelhoff et al., 1993; Walter et al., 1999). This is noteworthy since phosphorylation/dephosphorylation has been shown to be implicated in the regulation of many sorting proteins including the GGA proteins (Ghosh and Kornfeld, 2003). By this approach, we could show that phosphorylation of BACE1 in the cytoplasmic domain regulates the retrograde transport from endosomes to the TGN, while the reinternalization of BACE1 from the cell surface appears to occur independent of the phosphorylation state. While the S498A mutant that lacks the phosphorylation site was predominantly localized in vesicular structures even after longer incubation times, wild-type and the S498D mutant that mimic phosphorylated BACE1 were transported from early endosomal compartments to juxtanuclear structures, which were identified as the TGN. In accordance with this, the direct recycling of the S498D mutant from endosomal compartments back to the cell surface was much less efficient as compared to the S498A mutant. Taken together, our data suggest that non-phosphorylated BACE1 remains in endosomal compartments to juxtanuclear structures, which were identified as the TGN. In accordance with this, the direct recycling of the S498D mutant from endosomal compartments back to the cell surface was much less efficient as compared to the S498A mutant. Taken together, our data suggest that non-phosphorylated BACE1 remains in endosomal compartments that allow direct recycling to the plasma membrane, while phosphorylated BACE1 is targeted to transport vesicles destined to the TGN (Fig. 7).

Our data strongly suggest that the phosphorylation-dependent retrograde transport of BACE1 from endosomal compartments to the TGN is mediated by GGA proteins. First, phosphorylation of BACE1 results in increased binding of GGA1 to the cytoplasmic domain of BACE1 and efficient transport from endosomes to the...
TGN. Second, BACE1 is internalized from the cell surface into endosomal compartments where it colocalizes with GGA1, and third, expression of a dominant-negative form of GGA1 (GGA1 DN) inhibits the retrograde transport of BACE1 from endosomes to the TGN. Since GGA1 DN binds to the cytoplasmic domain of BACE1 via the VHS domain, but not to coat proteins on target vesicles due to the lack of the other functional domains (hinge region and GAE), we speculate that expression of this variant un couples the sorting of BACE1 from endosomes to transport vesicles destined to the TGN. The involvement of GGA proteins in retrograde trafficking of cargo proteins from endosomes to the TGN is further supported by the previous finding that down-regulation of GGA proteins leads to accumulation of MPRs in endosomal compartments (Ghosh et al., 2003b). Thus, GGA proteins might function in the sorting of cargo proteins between the TGN and endosomal compartments in both anterograde and retrograde direction, very similar to AP-1 (Hinners and Tooze, 2004).

It has been shown that the generation of Aβ involves BACE1-mediated cleavage of βAPP in endocytic compartments (Ehehalt et al., 2003; Haass et al., 1992; Koo and Squazzo, 1994). Since BACE1 has an acidic pH optimum, endosomal/lyosomal compartments might be the compartments with highest BACE1 activity and be the major sites for amyloidogenic processing of βAPP (Vassar and Citron, 2000; Walter et al., 2001a). As demonstrated here, the endocytic trafficking of BACE1 is mediated by GGA1 and possibly by other members of the GGA protein family. Consistent with previous studies (He et al., 2005; von Arnim et al., 2004), the secretion of APPs and Aβ was not significantly altered in cells overexpressing APP and BACE1 together with GGA wt or the DN variant (not shown). However, these data do not rule out the possibility that changes in the expression or the activity of GGA proteins at the endogenous level might result in the modulation of BACE1 activity and the generation of Aβ. It will therefore be interesting to analyze the role of GGA proteins in the pathogenesis of Alzheimer’s disease.

Fig. 7. Model of phosphorylation-dependent subcellular transport of BACE1. BACE1 is transported from the ER via the Golgi to the cell surface from where it is re-internalized into endosomes. Phosphorylation of its C-terminal serine residue 498 enhances its interaction with GGA proteins and the retrograde transport to the TGN, while lack of phosphorylation leads to recycling of BACE1 to the cell surface. In addition, there might exist a direct transport route from the TGN to endosomal compartments (marked by ?).

Experimental methods

Antibodies, cDNAs and cell culture

The monoclonal antibody against c-myc was purchased from LGC Promochem Inc. Antibodies 7520 and 7523 recognizing the C-terminus and the N-terminus of BACE1, respectively, have been described earlier (Walter et al., 2001b). Antibodies against EEA1 and TGN46 were obtained from BD Bioscience and Serotec Inc., respectively. The cell lines stably overexpressing βAPP and BACE1 wt or its phosphorylation site mutants S498A, S498D, have been described previously (Walter et al., 2001b). cDNAs encoding GGA proteins were provided by Drs. M. Robinson and J.S. Bonifacino and subcloned into pcDNA4/myc/his expression vector (Invitrogen). To generate fusion proteins of glutathione-S-transferase (GST) or maltose-binding-protein (MBP), the VHS domain of GGA1 was amplified by PCR, and the resulting fragment was subcloned into EcoRI/SalI restriction sites of pGEX-5X-1 (Amersham Pharmacia Biotech) or pmalC2 (New England Biolabs). Fusion proteins were expressed in Escherichia coli DH5α and purified according to the suppliers’ instructions. Cloning of BACE CT fusion proteins was described earlier (Walter et al., 2001b).

Pull-down assays, immunoprecipitation and Western immunoblotting

For pull-down assays, cells were lysed in STEN buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA) supplemented with 1% NP-40/1% Triton X-100/2% BSA on ice for 10 min. Lysates were clarified by centrifugation for 20 min at 14,000 × g and incubated with pre- incubated GSH-sepharose/GST-fusion protein (5 μg) for 2 h at 4°C. After separation by SDS-PAGE, proteins were transferred to nitrocellulose-membrane (Schleicher and Schüll Inc.) and analyzed by immunoblotting using enhanced chemiluminescence technique (Amersham Pharmacia Biotech).

Cell surface biotinylation and recycling assays

Cells were washed three times with ice-cold PBS and incubated on ice with PBS containing 0.5 mg/ml EZ-link™ Sulfo-NHS-SS-Biotin (Perbio) for 30 min. Cells were then washed three times with ice-cold PBS supplemented with 20 mM glycine for 5 min each and finally washed twice with PBS. To allow reinternalization, cells were then incubated with DMEM (10% fetal calf serum) at 37°C for 15 min. Subsequently, cells were washed three times with glutathione buffer (50 mM glutathione; 90 mM NaCl, 1.25 mM CaCl2, 1.25 mM MgSO4, 0.2% BSA, pH 8.6) for 15 min each. Under these conditions, the biotin-label of cell surface proteins is removed, while that of internalized proteins is protected against cleavage. After two final washes with PBS, cells were lysed in buffer containing 1% NP-40/1% Triton X-100. Precipitated BACE1 was detected by Western immunoblotting with antibody 7523, and endocytosis was quantified by detection with 125I-labeled secondary antibody and phosphoimaging.

For recycling assays, biotinylated proteins were allowed to internalize for 15 min at 37°C. Cells were then incubated with glutathione buffer to remove the biotin-label from proteins that remained at the cell surface. Cells were then incubated for 30 min at 37°C to allow recycling to the cell surface and washed again with ice-cold glutathione buffer. Biotinylated proteins were
isolated by precipitation with streptavidin-conjugated sepharose (Amersham Pharmacia Biotec). Precipitated BACE1 was detected by Western immunoblotting and quantified by detection with 125I-labeled secondary antibody and phosphoimaging. Statistical analysis was carried out by one-way ANOVA with Bonferroni’s multiple comparison test.

**Antibody uptake assay**

Cells grown on polylysine-coated glass cover slips were washed twice with ice-cold PBS and incubated for 30 min on ice with ice-cold PBS containing the indicated antibody. Cells were then washed three times with ice-cold PBS and subsequently incubated at 37°C in DMEM containing 10% fetal calf serum for various time periods. After two washes with PBS, cells were fixed in 4% paraformaldehyde and processed for immunofluorescence.

**Immunocytochemistry**

Cells were grown on polylysine-coated glass coverslips to 50–80% confluence and fixed in 4% paraformaldehyde at room temperature before processing for immunofluorescence as described previously (Walter et al., 2001b). Bound primary antibodies were detected by Alexa 488- or Alexa 594-conjugated secondary antibodies (Molecular Probes, Inc., Eugene, USA). Cells were analyzed using either Nikon fluorescence microscope or with an inverted confocal laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) equipped with an argon and a helium/neon mixed gas laser with excitation wavelengths of 488 or 543 nm. Scans at a resolution of 1024 x 1024 pixels were taken in the line-averaging mode and at a pinhole setting of one airy unit. Micrographs were stored in LSM or TIFF format (Zeiss LSM Image Browser version 2.30.011; Carl Zeiss Jena GmbH).

**Surface plasmon resonance measurements**

Synthetic biotin-coupled peptides representing the cytoplasmic tail of BACE1 in non-phosphorylated (biotin-CQWRCL-RCLRQQHDDFADDISLLK) or phosphorylated state (biotin-CQWRCLRCLRQQHDDFADDIS(P)LLK) were immobilized on a Biacore streptavidin chip using standard coupling according to the manufacturer’s instructions. Binding curves for GST-GGA1 VHS or GST (concentrations ranging from 50 nM to 5 μM) were obtained, and dissociation constants were determined by steady-state affinity curve fits.

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While the manuscript was in preparation, we learned that similar results have been obtained by He et al. with another cell type (He et al., 2005).

**References**


